DESCRIPTION

NOVEL POLYPEPTIDES AND POLYNUCLEOTIDES
RELATING TO THE α- AND β-SUBUNITS OF GLUTAMATE
DEHYDROGENASES AND METHODS OF USE

This invention was made with government support under USDA Competitive Grant Number 87-CRCR-1-2476. The government has certain rights in this invention.

Cross-Reference to a Related Application

This application is a continuation application of co-pending application Serial No. 09/070,844, filed May 1, 1998 which is a divisional application of co-pending application Serial No. 08/725,596, filed October 3, 1996 (now abandoned), which is a continuation-in-part of co-pending application Serial No. 08/541,033, filed October 6, 1995 (now U.S. Patent No. 5,879,941, issued March 9, 1999).

Background of the Invention

Inorganic nitrogen acquired by plants is ultimately converted to ammonium before being assimilated in organic nitrogen metabolism. One enzyme postulated to be involved in the assimilatory process is glutamate dehydrogenase (GDH), a group of ubiquitous enzymes found to be present in almost all organisms from microbes to higher plants and animals (Srivastava, H.S., R.P. Singh [1987] *Phytochem.* 26:597-610). GDH catalyses the reversible conversion of α-ketoglutarate to glutamate via a reductive amination that utilizes reduced β-nicotinamide adenine dinucleotide (NADH) or reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The role of plant GDHs in the assimilation of ammonium into amino acids has been questioned since the discovery of the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway that is believed to be the favored pathway for ammonium assimilation in higher plants (Miflin, B.J., P.J. Lea [1976] *Phytochem.* 15:873-885).

The primary objection to GDH playing a major role in plant nitrogen metabolism is its low affinity for ammonium that would require high intracellular ammonium concentrations to function anabolically. Early evidence indicated that GDH is a catabolic enzyme catalyzing the deamination of glutamate with only a partially anabolic function in

5   synthesizing glutamate (Wallgrove, J.C., N.P. Hall, A.C. Kendall, [1987] *Plant Physiol.* 83:155-158). The physiological role of large amounts of GDH present in various plant tissues and organelles is still unclear, and possible conditions under which GDH may play a significant role in carbon and nitrogen metabolism have not been resolved.

The majority of plant GDHs characterized to date are localized in the mitochondria;

10   however, a GDH species differing in several properties (e.g., cofactor specificity, K_m values, organelle localization, thermal stability, among others) has been characterized from the chloroplast of a unicellular green alga *Chlorella sorokiniana*. *C. sorokiniana* cells have been shown to possess a constitutive, mitochondrial, tetrameric NAD-specific GDH (hereinafter designated "NAD-GDH") (Meredith, M.J., R.M. Gronostajski, R.R. Schmidt [1978] *Plant*

15   *Physiol.* 61:967-974), and seven ammonium-inducible, chloroplast-localized, homo- and heterohexameric NADP-specific GDH isoenzymes (hereinafter designated "NADP-GDH")(Prunkard, D.E., N.F. Bascomb, R.W. Robinson, R.R. Schmidt [1986] *Plant Physiol.* 81:349-355; Bascomb, N.F., R.R. Schmidt [1987] *Plant Physiol.* 83:75-84). The seven chloroplastic NADP-GDH isoenzymes were shown to have different electrophoretic

20   mobilities during native-PAGE, which can result from the formation of homo- and heterohexamers composed of varying ratios of α- and β-subunits (53.5 and 52.3 kilodaltons, respectively).

Chlorella cells cultured in 1 to 2 mM ammonium medium accumulate only the α-homohexamer (Bascomb and Schmidt, *supra*). The addition of higher ammonium

25   concentrations (3.4 to 29 mM) to nitrate-cultured cells results in the accumulation of both α- and β-subunits in NADP-GDH holoenzymes (Prunkard *et al.*, *supra*; Bascomb and Schmidt, *supra*; Bascomb, N.F., D.E. Prunkard, R.R. Schmidt [1987] *Plant Physiol.* 83:85-91). Prunkard *et al.* (Prunkard, D.E., N.F. Bascomb, NF, W.T. Molin, R.R. Schmidt [1986] *Plant Physiol.* 81:413-422) demonstrated that the NADP-GDH subunit ratio and isoenzyme

pattern is influenced by both the carbon and nitrogen source as well as the light conditions under which cells are cultured.

The α- and β-NADP-GDH homohexamers purified from *Chlorella* cells have strikingly different ammonium K_m values; however, the K_m values for their other substrates are very similar. The α-homohexamer (composed of six identical α-subunits) that catalyzes the biosynthesis of glutamate is allosterically regulated by NADPH and possesses an unusually low K_m for ammonium that ranges from 0.02 to 3.5 mM, depending on the NADPH concentration (Bascomb and Schmidt, *supra*). The K_m value for ammonium of the α-homohexamer is the lowest reported ammonium K_m for any plant GDH characterized to date. In contrast, the β-homohexamer (catabolic form) is a non-allosteric enzyme with an ammonium K_m of approximately 75 mM. From these studies involving purified enzymes, it had been heretofore postulated that the heterohexamers have varying degrees of affinity for ammonium ranging between the K_m values for the α- and β-homohexamers. Surprisingly, however, we have discovered that certain heterohexamers can have aminating:deaminating activity ratio which is greater than either the α- or β-homohexamers.

Although the α- and β-subunits have distinct *in vivo* turnover rates (Bascomb *et al.*, *supra*) and the corresponding homohexamers have remarkably different ammonium K_m values, the α- and β-subunits are derived from precursor proteins of nearly identical size (ca 58,000 Daltons) and were shown to have very similar peptide maps (Prunkard *et al.*, *supra*; Bascomb and Schmidt, *supra*). Moreover, polyclonal antibodies prepared against the β-homohexamer are capable of immunoprecipitating all of the NADP-GDH isoenzymes - (Yeung, A.T., K.J. Turner, N.F. Bascomb, R.R. Schmidt [1981] *Anal. Biochem.* 10:216-228; Bascomb *et al.*, *supra*), but do not crossreact with the mitochondrial NAD-GDH. In addition, previous research in this laboratory provided genomic cloning and southern blot evidence that indicated the *C. sorokiniana* genome possesses a single NADP-GDH structural gene (Cock, J.M., K.D. Kim, P.W. Miller, R.G. Hutson, R.R. Schmidt [1991] *Plant Mol. Biol.* 17:17-27).

The *C. sorokiniana* nuclear-encoded chloroplastic NADP-GDH isoenzymes are the only chloroplastic localized GDH sequences isolated and characterized from plants.

Although the *Chlorella* GDH isoenzymes had been previously characterized, it has been discovered in the present invention that the two mature subunits arise via specific processing of two similar precursor proteins encoded by two mRNAs formed by alternative splicing of a pre-mRNA derived from a single nuclear gene. Furthermore, the identification of the

5   cleavage site and amino-terminal peptide sequence of the mature functional GDH subunits had not been accomplished prior to the present invention.

Brief Summary of the Invention

The present invention provides the isolation and characterization of two full-length

10   cDNAs from mRNAs isolated from the unicellular green algae *Chlorella sorokiniana*. The two cDNAs encode the precursor proteins (α-precursor, 56.35 kD; β-precursor, 57.85 kD) that are processed to yield the mature α- and β-subunits (53.5 kD; 52.3 kD, respectively) that compose the active NADP-GDH hexameric isoenzymes. The present invention concerns a single NADP-GDH gene which is alternatively spliced to yield two mRNAs that encode two

15   different chloroplast precursor proteins. These precursor proteins can then be processed to the mature α- and β-subunits of the NADP-GDH isoenzymes. Also described are useful fragments or mutants of the nucleotide and amino acid sequences which retain the disclosed activity or utility. For example, certain fragments of the amino acid sequences provided herein can be useful as transit peptides, providing the protein with the capability to enter and

20   remain in certain cell compartments. The nucleotide sequences which are described herein, and fragments of those nucleotide sequences, can be useful, for example, as primers in amplification procedures or as probes to hybridize to complementary sequences of interest. The nucleotide and amino acid sequences and fragments thereof as described herein can also be useful as molecular weight markers or in identifying and conforming the relatedness of

25   other nucleotide sequences, polypeptides, or isoenzymes which pertain to NADP-GDH.

The present invention further provides methods in which assimilation of inorganic nitrogen into organic nitrogen metabolism of higher plants can be altered by expressing GDH from *C. sorokiniana* or GDHs isolated from other organisms. The alteration of nitrogen assimilation can have the effect of increasing nitrogen assimilation which, as is well

understood in the art, can affect the composition of the plant through an inverse effect on carbon metabolism, e.g., accumulation of carbohydrates. The subject invention also concerns DNA constructs for use in the described methods. The present invention includes the identification of the amino-terminal sequences of the α- and β-subunits which can assemble

5   to form NADP-GDH isoenzymes, e.g., the native hexameric NADP-GDH found in *C. sorokiniana* chloroplasts. This precise molecular information can be employed to express NADP-GDH with the unique kinetic properties of the *C. sorokiniana* chloroplastic α- and β-NADP-GDH homohexamers. The present invention also provides recombinant cells or organisms, e.g., transgenic crops or plants which, by expressing the genes of the described

10   polynucleotide sequences to produce corresponding polypeptides, can have an increased yield, improved ammonia assimilatory properties which can advantageously increase their tolerance of ammonia toxicity, improved osmotic stress tolerance, and improved composition of the crop or plant.

15   Brief Description of the Drawings

Figure 1 shows a pattern of NADP-GDH activities in homogenates of synchronous *C. sorokiniana* cells cultured for 240 min in 29 mM ammonium medium in continuous light. Aliquots of clarified homogenates, from cell collected at various time intervals, were analyzed spectrophotometrically for both aminating (●) and deaminating (○) NADP-GDH

20   activities.

Figure 2 shows patterns of accumulation of NADP-GDH antigens in illuminated cells cultured in 29 mM ammonium medium for 240 min. At zero time, ammonium was added to synchronous *C. sorokiniana* daughter cells and the culture was illuminated. Autoradiographs of Western blots were analyzed by laser densitometry to determine the

25   relative levels of the NADP-GDH α-subunit (●) and β-subunit (○) throughout the 240 min induction period.

Brief Description of the Sequences

SEQ ID NO. 1 is the cDNA for the precursor-protein of the α-subunit of an NADP-specific glutamate dehydrogenase.

SEQ ID NO. 2 is the deduced amino acid sequence of the polynucleotide of SEQ ID NO. 1.

SEQ ID NO. 3 is the cDNA for the precursor-protein of the β-subunit of an NADP-specific glutamate dehydrogenase.

SEQ ID NO. 4 is the deduced amino acid sequence of the polynucleotide of SEQ ID NO. 3.

SEQ ID NO. 5 is the N-terminal sequence for the NADP-GDH α-subunit.

SEQ ID NO. 6 is the N-terminal sequence for the NADP-GDH β-subunit.

SEQ ID NO. 7 is the cDNA sequence in the clone designated pBGDc53.

SEQ ID NO. 8 is a primer which hybridizes to the conserved region of NADP-GDH mRNAs.

SEQ ID NO. 9 is a poly(dT) polynucleotide used as an adaptor primer according to the subject invention.

SEQ ID NO. 10 is a polynucleotide used as a primer according to the subject invention.

SEQ ID NO. 11 is a polynucleotide used as a primer according to the subject invention.

SEQ ID NO. 12 is a polynucleotide used as an adaptor primer according to the subject invention.

SEQ ID NO. 13 is the polynucleotide insert in the clone designated pRGDc 60.

SEQ ID NO. 14 is the polynucleotide insert in the clone designated pRGDc 61.

SEQ ID NO. 15 is the polynucleotide used as a primer according to the subject invention.

SEQ ID NO. 16 is the polynucleotide insert in a clone designated pGDc 63.

SEQ ID NO. 17 is the polynucleotide insert of a clone designated pGDc 64.

SEQ ID NO. 18 is the polynucleotide resulting from ligation of purified fragments of the inserts in the clones designated pBGDc 53 and pGDc 63, according to the subject invention.

SEQ ID NO. 19 is the polynucleotide resulting from ligation of purified inserts of the clones designated pGDc 64 and pBGDc 53.

SEQ ID NO. 20 is a polynucleotide used as a primer according to the subject invention.

SEQ ID NO. 21 is a polynucleotide used as a primer hybridizing to the 3′ terminus of the template DNA according to the subject invention.

SEQ ID NO. 22 is a polynucleotide used as a primer according to the subject invention.

SEQ ID NO. 23 is the polynucleotide sequence (cDNA) of the processed, mature NADP-GDH α-subunit.

SEQ ID NO. 24 is the amino acid sequence of the processed, mature NADP-GDH α-subunit.

SEQ ID NO. 25 is the polynucleotide (cDNA) sequence of the processed, mature NADP-GDH β-subunit.

SEQ ID NO. 26 is the amino acid sequence of the processed, mature NADP-GDH β-subunit.

Detailed Disclosure of the Invention

The present invention provides heretofore undescribed polynucleotide sequences, for example, cDNAs for precursor-proteins of α- and β-subunits of an ammonium inducible, chloroplast-localized NADP-specific glutamate dehydrogenase (hereinafter NADP-GDH) from *Chlorella sorokiniana*. The nucleotide sequences for the precursor proteins of the α- and β-subunits that form NADP-GDH are shown in SEQ ID NOS. 1 and 3, respectively. The deduced amino acid sequences for the precursor-proteins of the α- and β-subunits of the NADP-GDH enzyme from *Chlorella sorokiniana* are shown in SEQ ID NOS. 2 and 4, respectively.

E. coli hosts comprising the subject cDNA inserts were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 USA. The cultures were assigned the following accession numbers by the repository:

	Culture	Accession number	Deposit date
5	*E. coli* DH5α α-NADP-GDH SEQ No. 1 (+42 bp)	ATCC 69925	October 6, 1995
10	*E. coli* DH5α β-NADP-GDH SEQ No. 1 (−42 bp)	ATCC 69926	October 6, 1995

The subject cultures have been deposited under conditions that assure that access to the culture(s) will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of a deposit(s), and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposit(s) should the depository be unable to furnish a sample when requested, due to the condition of the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Automated amino acid sequence analysis identifies 20 and 10 amino-terminal amino acid residues of the α- and β-subunits, respectively. Alignment of the α- and β-subunit peptide sequences reveals that the two subunits are identical with the exception of an 11-amino acid extension present in the larger α-subunit. Monoclonal antibodies raised against the α-subunit were shown to recognize the β-subunit providing further evidence that the two subunits are nearly identical. The identification of the unique α- and β-subunit processing sites within the precursor proteins provides the molecular mechanism to explain the different kinetic properties of the α- and β-NADP-GDH homohexameric isoenzymes.

The aforementioned data provide information applicable to genetically engineer plants with a specific GDH having favorable kinetic properties which can influence both carbon and nitrogen metabolism. Based on the high guanine/cytosine content the cDNAs are highly amenable for heterologous expression in higher plants. The introduction of either or both subunits with their chloroplast targeting sequences or with other organellar targeting sequences in heterologous plant systems can improve nitrogen assimilation and influence the carbon/nitrogen balance.

It has been discovered that chloroplast localization is related to, and can be dependent on, the N-terminus of the α- or β-precursor protein. Cleavage of the N-terminus of the precursors yields the mature proteins. Accordingly, the chloroplast transit peptide comprises a peptide which forms, or is an active fragment of, the N-terminus cleaved from the precursor protein. Peptides having similar or equivalent amino acid sequences, or that have a tertiary structure or conformation similar to these cleaved peptides can also function as transit peptides. The chloroplast-transit peptide comprises the active fragment of the N-terminal peptide cleaved from the α-precursor (a 40-mer) or the β-precursor (a 37-mer). The polynucleotide sequences encoding the chloroplast-transit peptides can be used by persons of ordinary skill in the art to produce chloroplast-transit peptides employed with the peptides described herein, or others known in the art.

Adding, removing, or replacing the chloroplast-transit peptide associated with a protein, e.g., the GDH enzyme, can be employed to localize the protein according to need, by means well known in the art. For example, localization of the enzyme in a chloroplast of

a cell can be achieved by the insertion of a chloroplast-transit peptide onto an amino acid sequence lacking such a transit peptide. Species-specific chloroplast-transit peptides can be added or can replace those present to optimize insertion into the chloroplast of a desired species. In addition, localization inside the chloroplast of a protein expressed within the

5   chloroplast can be achieved by direct transformation of the plastid with the polynucleotide sequences encoding an expressed protein. Similarly, removal of a chloroplast-transit peptide or production of a recombinant protein lacking the peptide can be utilized to sequester the protein in a cellular compartment other than the chloroplast.

Transformed plants expressing the α-homohexamer can be more tolerant to ammonia

10   toxicity, assimilate ammonium more efficiently, and respond more rapidly to osmotic stress encountered in transiently saline soils by providing glutamate the precursor to the osmoprotectant proline. Expression of, for example, the β-homohexamer or GDH heterohexamers can be used to alter the rate of nitrogen assimilation, favoring accumulation of carbohydrates in fruits and other storage organs.

15   Unexpectedly, it was discovered that a hexamer comprising at least one α-subunit and at least one β-subunit, i.e., a heterohexamer, can have advantageous activity. Specifically, the aminating:deaminating activity ratio (i.e., biosynthetic capacity for synthesis of glutamate) of a chloroplastic NADP-GDH isozyme can be increased by incorporating both α- and β-subunits into the hexameric protein rather than using a homohexamer comprising

20   only the α- or only the β-subunits. In one embodiment of the invention, it can be advantageous to co-express cDNAs encoding both types of subunits in the same plant at different rates/levels such that a particular ratio of α- and β-subunits is obtained in the heterohexamer. For example, we have discovered that an NADP-GDH heterohexamer having at least one of the subunits in the β-form is preferred for increasing

25   aminating:deaminating activity ratio. A more preferred heterohexamer has 2–5 β-subunits. This differential rate of expression of the two cDNAs can be accomplished by placing them under the control of plant promoters with different strengths or under the same promoter that has been modified to generate different levels of expression. The use of this algal NADP-

GDH isozyme system in plant biotechnology has advantages over NADP-GDHs from organisms, such as bacteria, that contain only a single form of the enzyme (i.e., no isozymes).

It is recognized that expression levels of certain recombinant proteins in transgenic plants can be improved via increased expression of stabilized mRNA transcripts; and that,

5   conversely, detection of these stabilized RNA transcripts may be utilized to measure expression of translational product (protein). Low expression of protein RNA in plants and, therefore, of low protein expression, can be resolved through the use of an improved, synthetic gene specifying the desired protein from the gene source organism.

Thus, in one embodiment of the subject invention, bacteria and plants can be

10   genetically engineered to attain desired expression levels of novel proteins having agricultural or otherwise commercial value. To provide genes having enhanced expression in plants, the DNA sequence of the gene can be modified to comprise codons preferred by highly expressed plant genes, to attain an A+T content in nucleotide base composition substantially that found in plants, and also preferably to form a plant initiation sequence, and

15   to eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA and to avoid sequences that constitute secondary structure hairpins and RNA splice sites. For example, in synthetic genes, the codons used to specify a given amino acid can be selected with regard to the distribution frequency of codon usage employed in highly expressed plant genes to specify that amino acid. As is appreciated by

20   those skilled in the art, the distribution frequency of codon usage utilized in the synthetic gene is a determinant of the level of expression.

For purposes of the subject invention, "frequency of preferred codon usage" refers to the preference exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. To determine the frequency of usage of a particular codon in a gene, the

25   number of occurrences of that codon in the gene is divided by the total number of occurrences of all codons specifying the same amino acid in the gene. Similarly, the frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell. It is preferable that this analysis be limited to genes that are highly expressed by the host cell.

When synthesizing a gene for improved expression in a host cell it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

The percent deviation of the frequency of preferred codon usage for a synthetic gene from that employed by a host cell is calculated first by determining the percent deviation of the frequency of usage of a single codon from that of the host cell followed by obtaining the average deviation over all codons. As defined herein this calculation includes unique codons (i.e., ATG and TGG). In general terms the overall average deviation of the codon usage of a synthetic gene from that of a host cell is calculated using the equation

$$A = \sum_{n=1}^{Z} \frac{\frac{X_n - Y_n}{X_n} \times 100}{Z}$$

where X_n=frequency of usage for codon n in the host cell; Y_n=frequency of usage for codon n in the synthetic gene. Where n represents an individual codon that specifies an amino acid, the total number of codons is Z. The overall deviation of the frequency of codon usage, A, for all amino acids should preferably be less than about 25%, and more preferably less than about 10%. Hence, a gene can be designed such that its distribution frequency of codon usage deviates, preferably, no more than 25% from that of highly expressed plant genes and, more preferably, no more than about 10%. In addition, consideration is given to the percentage G+C content of the degenerate third base (monocotyledons appear to favor G+C in this position, whereas dicotyledons do not). It is also recognized that the XCG (where X is A, T, C or G) nucleotide is the least preferred codon in dicots whereas the XTA codon is avoided in both monocots and dicots. Synthetic genes of this invention also preferably have CG and TA doublet avoidance indices closely approximating those of the chosen host plant. More preferably these indices deviate from that of the host by no more than about 10-15%.

Assembly of the NADP-GDH gene of this invention can be performed using standard technology known in the art. A structural gene designed for enhanced expression in plants of the specific embodiment can be enzymatically assembled within a DNA vector from chemically synthesized oligonucleotide duplex segments. The gene can then be introduced into a plant host cell and expressed by means known to the art. Preferably, the protein produced upon expression of the synthetic gene in plants is functionally equivalent to a native protein in having comparable or improved aminating/deaminating activity. According to the subject invention, *functionally equivalent* refers to identity or near identity of function. A synthetic gene product which has at least one property relating to its activity or function, which is the same or similar to a natural protein is considered functionally equivalent thereto.

Modifications in nucleotide sequence of the coding region can be made to alter the A+T content in DNA base composition of a synthetic gene to reflect that normally found in genes for highly expressed proteins native to the host cell. Preferably the A+T content of the synthetic gene is substantially equal to that of said genes for highly expressed proteins. In genes encoding highly expressed plant proteins, the A+T content is approximately 55%. It is preferred that the synthetic gene have an A+T content near this value, and not sufficiently high as to cause destabilization of RNA and, therefore, lower the protein expression levels. More preferably, the A+T content is no more than about 60% and most preferably is about 55%. Also, for ultimate expression in plants, the synthetic gene nucleotide sequence preferably can be modified to form a plant initiation sequence at the 5′ end of the coding region. In addition, particular attention is preferably given to assure that unique restriction sites are placed in strategic positions to allow efficient assembly of oligonucleotide segments during construction of the synthetic gene and to facilitate subsequent nucleotide modification. As a result of these modifications in coding region of the native gene, the preferred synthetic gene is expressed in plants at an enhanced level when compared to that observed with natural structural genes.

It is known that the relative use of synonymous codons differs between the monocots and the dicots. In general, the most important factor in discriminating between monocot and dicot patterns of codon usage is the percentage G+C content of the degenerate third base.

In monocots, 16 of 18 amino acids favor G+C in this position, while dicots only favor G+C in 7 of 18 amino acids.

For soybean and maize, the maize codon usage pattern resembles that of monocots in general, whereas the soybean codon usage pattern is almost identical to the general dicot pattern.

In designing a synthetic gene for expression in plants, it is preferred to eliminate sequences which interfere with the efficacy of gene expression.

A synthetic gene may be synthesized for other purposes in addition to that of achieving enhanced levels of expression. For example, in accordance with the subject invention, one of the nucleotide sequences encoding the α-subunit or the β-subunit of NADP-GDH can be modified such that the products are differentially expressed, favoring expression of one of the subunits. A result of such differential expression is a heterohexamer comprising more of one subunit than the other. Modification may encompass substitution of one or more, but not all, of the oligonucleotide segments used to construct the synthetic gene by a corresponding region of natural sequence. Preferably, differential expression of the nucleotide sequences encoding the α- and β-subunits of the NADP-GDH polypeptides can be employed to produce a heterohexamer having at least one β-subunit, more preferably two to five β-subunits, and most preferably three β-subunits.

The recombinant DNA molecule comprising a nucleotide sequence of the subject invention can be introduced into plant tissue by any means known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques. As novel means are developed for the stable insertion of foreign genes into plant cells and for manipulating the modified cells, skilled artisans will be able to select from known means to achieve a desired result. Means for introducing recombinant DNA into plant tissue include, but are not limited to, direct DNA uptake (Paszkowski, J. et al. (1984) EMBO J. 3:2717), electroporation (Fromm, M. et al. (1985) Proc. Natl. Acad. Sci. USA 82:5824), microinjection (Crossway, A. et al. (1986) Mol. Gen. Genet. 202:179), or T-DNA mediated transfer from *Agrobacterium tumefaciens* to the plant tissue. There appears to be no fundamental limitation of T-DNA transformation to the

natural host range of Agrobacterium. Successful T-DNA-mediated transformation of monocots (Hooykaas-Van Slogteren, G. et al. (1984) Nature 311:763), gymnosperms (Dandekar, A. et al. (1987) Biotechnology 5:587) and algae (Ausich, R., EPO application 108,580) has been reported. Representative T-DNA vector systems are described in the following references: An, G. et al. (1985) EMBO J. 4:277; Herrera-Estrella, L. et al. (1983) Nature 303:209; Herrera-Estrella, L. et al. (1983) EMBO J. 2:987; Herrera-Estrella, L. et al. (1985) in *Plant Genetic Engineering*, New York: Cambridge University Press, p. 63. Once introduced into the plant tissue, the expression of the structural gene may be assayed by any means known to the art, and expression may be measured as mRNA transcribed or as protein synthesized. Techniques are known for the in vitro culture of plant tissue, and in a number of cases, for regeneration in to whole plants. Procedures for transferring the introduced expression complex to commercially useful cultivars are known to those skilled in the art.

In one of its preferred embodiments the invention disclosed herein comprises expression in plant cells of an NADP-GDH gene under control of a plant expressible promoter, that is to say, by inserting the gene into T-DNA under control of a plant expressible promoter and introducing the T-DNA containing the insert into a plant cell using known means. Once plant cells expressing the gene under control of a plant expressible promoter are obtained, plant tissues and whole plants can be regenerated therefrom using methods and techniques well-known in the art. The regenerated plants are then reproduced by conventional means and the introduced genes can be transferred to other strains and cultivars by conventional plant breeding techniques.

The introduction and expression of the NADP-GDH gene can be used to improve, e.g., increase, yields in a crop. Other uses of the invention, exploiting the properties of the genes introduced into plant species will be readily apparent to those skilled in the art.

Differences also exist between codon choice in plant nuclear genes and in cholorplasts. Chloroplasts differ from higher plants in that they encode only 30 tRNA species. Since chloroplasts have restricted their tRNA genes, the use of preferred codons by chloroplast-encoded proteins appears more extreme. However, a positive correlation has been reported between the level of isoaccepting tRNA for a given amino acid and the

frequency with which this codon is used in the chloroplast genome (Pfitzinger et al. (1987) Nucl. Acids Res. 15:1377-1386. In general, the chloroplast codon profile more closely resembles that of unicellular organisms, with a strong bias towards the use of A+T in the degenerate third base.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLES

Example 1 – Kinetics of C. sorokiniana Chloroplast Glutamate Dehydrogenases

The chloroplastic glutamate dehydrogenase α- and β-isoenzymes used in the following experiments are naturally produced by an organism characterized as *Chlorella sorokiniana.*

C. sorokiniana culture conditions. For kinetic characterization in both the aminating and deaminating directions, the α- and β-holoenzymes were purified from cells that were accumulating only one form of homohexameric GDH isoenzyme.

The *C. sorokiniana* cells (UTEX-1230, University of Texas algal culture collection; 3B2NA, Robert R. Schmidt, University of Florida, Microbiology Cell Science Department) were cultured autotrophically as previously described by Prunkard *et al., supra* in a modified basal salts medium. The modified medium contained in mM concentration: $CaCl_2$, 0.34; K_2SO_4, 6.0; KH_2PO_4, 18.4; $MgCl_2$, 1.5; in μM concentration $CoCl_2$, 0.189; $CuCl_2$, 0.352; EDTA, 72; $FeCl_3$, 71.6; H_3BO_3, 38.8; $MnCl_2$, 10.1; NH_4VO_4, 0.20; $(NH_4)_6MO_7O_{24}$, 4.19; $NiCl_2$, 0.19; $SnCl_2$, 0.19; $ZnCl_2$, 0.734. The medium was supplemented with 1 mM NH_4Cl, 29 mM NH_4Cl, or 29 mM KNO_3 as a nitrogen source depending on the experimental conditions. The medium containing NH_4Cl was adjusted to pH 7.4, and medium containing KNO_3 was adjusted to pH 6.8 with KOH after autoclaving. Cells were supplied with a 2% (v/v) CO_2-air mixture and light intensity sufficient to allow cell division into four progeny.

Purification of the NADP-GDH isoenzymes. For purification of the glutamate dehydrogenase α-isoenzyme, *C. sorokiniana* cells were cultured with continuous light in 29 mM ammonium medium in a 30 L Plexiglas chamber as previously described (Baker, A.L., R.R. Schmidt [1963] *Biochim. Biophys. Acta* 74:75-83). Cells were harvested at 4.0 OD_{640}

5   by centrifugation at 30,000 rpm through a Sharples centrifuge and washed two times in 10 mM Tris (pH 8.5 at 4°C). Pelleted cells (130 g) were stored at −20°C in 250 mL centrifuge bottles until use. Purification of NADP-GDH was accomplished using a modified procedure of Yeung *et al., supra*. Procedural modifications involved the substitution of Sephadex G-200 gel (Pharmacia) for G-150 gel in the gel-filtration column, and the addition of $NADP^+$

10   as a stabilizer to a final concentration of 0.1 mM to the gel-filtration buffer and all subsequent storage buffers. As a final modification, the $NADP^+$ affinity resin step was omitted and a preparative nondenaturing-PAGE step was substituted (Miller, P.W., W.D. Dunn, R.R. Schmidt [1994] *BioRad US/EG Bulletin 1897*).

The GDH deaminating enzyme assay solution was composed of 44 mM Tris, 20.4

15   mM glutamate, and 1.02 mM $NADP^+$, pH 8.8. The aminating assay solution was composed of 50 mM Tris, 25 mM α-ketoglutarate, 0.357 mM NADPH, and 0.356 M $(NH_4)_2SO_4$, pH 7.4. One unit of enzyme activity was the amount of NADP-GDH required to reduce or to oxidize 1.0 μmol of $NADP^+$ or NADPH per minute at 38.5°C.

Sephadex G-200 column fractions possessing NADP-GDH activity were pooled and

20   concentrated via Diaflow filtration. The soluble enzyme (68 mg) was protected from oxidation by the addition of DTT to a final concentration of 10 mM, and dialyzed for 30 minutes against 28.8 mM Tris, 192 mM glycine, 2 mM DTT (pH 8.4). The dialysate was clarified by centrifugation at 20,000g for 10 minutes at 4°C and was combined with 3 mL of 40% (w/v) sucrose and 1 mL of 0.02% bromophenol blue.

25   For preparative nondenaturing PAGE, a 3 cm tall 7% acrylamide (w/v, 28 acrylamide: 0.735 bis-acrylamide, pH 8.8) resolving gel, and a 2 cm tall 2% acrylamide (w/v, 1.6 acrylamide: 0.4 bis-acrylamide, pH 6.6) stacking gel were cast in the 28 mm ID gel tube of the Model 491 Prep Cell. All acrylamide stocks were pretreated with AG501-X8 mixed bed resin to remove any contaminating acrylic acid residue to prevent *in vitro* N-acylation

of proteins during electrophoresis. The protein sample was electrophoresed at 15 mA constant power for 20 minutes and then for 3.5 hours at a constant power of 30 mA. Six milliliter fractions were collected and assayed for NADP-GDH deaminating activity and GDH containing fractions were pooled. The enzyme in the pooled fractions in 10 mM KPO_4

5   (pH 6.2), 0.1 mM $NADP^+$ was concentrated by Diaflow ultrafiltration to 1 mg/mL as determined by the method of Bradford, using BSA as a standard. The concentrated enzyme preparation was stored at $-20°C$. The purity of the preparation was determined by silver-staining to visualize proteins resolved by 10% (w/v) Tris-Tricine SDS-PAGE (Schagger, H., G. von Jagow [1987] *Anal. Biochem.* 166:368-379).

10   The NADP-GDH β-isoenzyme was purified from a mixture of cells cultured for 240 minutes in 1 mM ammonium medium (14 g), 90 minutes in 1 mM ammonium medium (6 g), and for 20, 40, 60, and 80 minutes in 29 mM ammonium medium (1 g/time point) according to Bascomb and Schmidt, *supra*. The NADP-GDH β-isoenzyme was partially purified using a scaled down modified procedure of Yeung *et al., supra*. The DEAE

15   sephacel ion exchange columns (pH 7.4, and pH 6) were scaled down to a 40 mL bed volume and a 400 mL linear KCl gradient (0 to 0.4 M) was used to elute the proteins in 3 mL fractions. The pH 6 DEAE ion-exchange column fractions containing NADP-GDH were combined into two pools; corresponding to the leading and trailing halves of the NADP-GDH activity peak. The separate pooled fractions were dialyzed against 10 mM KPO_4 (pH

20   6.2), 2 mM DTT for 16 hours, and affinity purified using Type 3 $NADP^+$ affinity gel (Pharmacia) as previously described (Bascomb and Schmidt, *supra*). The NADP-GDH in the pooled fractions was concentrated via Diaflow ultrafiltration to 2 mg/ml protein, as determined by the method of Bradford (Bradford, M.M. [1976] *Anal. Biochem.* 72:248-254), and stored at 4°C until further use. After resolution of the proteins by 8% (w/v) Tris-Tricine

25   SDS-PAGE, the purity of the preparation was determined by silver staining.

Table 1 summarizes the K_m values determined for both the α- and β-homohexameric isoenzyme aminating reaction.

Table 1		
GDH Isoform	Substrate	K_m Value (mM)
α-homohexamer	NADPH	0.14
	NH_4^+	0.02-3.5
	α-ketoglutarate	0.35*
β-homohexamer	NADPH	0.14
	NH_4^+	77
	α-ketoglutarate	12

*after Shatilov, V.R., W.L. Kretovich (1977) *Mol. Cell Biochem.* 15:201-212.

Table 2 summarizes the K_m values determine for both the α- and β-homohexameric isoenzyme deaminating reaction.

Table 2		
GDH Isoform	Substrate	K_m Value (mM)
α-homohexamer	$NADP^+$	0.04
	Glutamate	38.2
β-homohexamer	NADP+	0.04
	Glutamate	32.3

Activity of the α-, β-heterohexamer. The aminating and deaminating activities of the mixture of native NADP-GDH isoenzymes (heterohexamers composed of varying ratios of the α- and β-subunits) were also measured with saturating levels of substrates throughout the 240 minute induction period (Fig. 1). The aminating and deaminating activities showed initial induction lags of 20 to 40 min, respectively. The aminating activity increased rapidly during the first 100 min, decreased sharply between 100 min and 140 min, and increased

sharply once again between 140 min and 240 min. In contrast, the deaminating activity increased in almost a linear manner throughout the induction after the initial induction-lag.

During the 240 min induction period in 29 mM ammonium medium, the patterns of accumulation of the *Chlorella sorokiniana* NADP-GDH α- and β-subunits in isoenzymes were also examined by use of a western blot immunodetection procedure following SDS polyacrylamide-gel electrophoresis (see Fig. 2). The NADP-GDH β-subunit was detected at T_0 and increased for the first 40 min followed by a gradual decrease through the remainder of the induction period. The α-subunit was first detected at 20 min. This subunit accumulated at a low rate for the first 80 min, showed a marked increase between 80 min and 100 min, and thereafter accumulated in a linear manner at a lower rate for the remainder of the induction period. The transition from the β-subunit being the prominent species to the α-subunit being prominent occurred between 60 and 80 min.

The aminating:deaminating activity ratio and the α:β subunit ratio were calculated to determine if changes in the subunit ratio in the mixture of NADP-GDH isoenzymes correlated with the predicted aminating:deaminating activity ratio during the time-course of the induction period (Table 3). Surprisingly, the highest aminating:deaminating ratio was observed at 60 min when the subunit ratio showed the β-subunit to be the prominent NADP-GDH antigen, whereas the α-subunit was the prominent form when the aminating:deaminating activity ratio was the lowest. This latter result was not predictable in advance.

Prior to this discovery, substrate kinetic studies of purified α- and β-homohexamers, the α-homohexamer, with its very high affinity for ammonium (relative to the β-homohexamer), was assumed to be the isoenzyme-form with the highest aminating activity (i.e., biosynthetic capacity for glutamate synthesis). The results suggested that the individual subunits would act independently with respect to their kinetic properties in homo- and heterohexamers.

Comparison of the aminating:deaminating activity ratio with the α:β subunit ratio throughout the 240 min induction in 29 mM ammonium medium revealed an unexpected correlation between the maxima in these ratios (Table 3).

Table 3. NADP-GDH aminating :deaminating activity and α-subunit:β-subunit ratios during ammonium induction period in *C. sorokiniana* cells.

Table 3		
Time (min)	Am:Deam Activity	α:β Subunit
0	2.87	0.28
20	2.96	0.58
40	3.81	0.49
60	4.51	0.80
80	3.49	1.57
100	2.73	8.74
140	1.61	11.23
240	1.12	24.70

The peak in aminating:deaminating ratio occurred at 60 min at which time the β-subunit was the prominent but not exclusive antigen, whereas the α-subunit was prominent when the aminating:deaminating ratio was lowest. Interestingly, the aminating activity was highest when both subunits were present, suggesting that heterohexamer(s), formed by combination(s) of the α- and β-subunits, can have a higher aminating activity than a homohexamer. Based on the much lower K_m of the purified α-homohexamer that the β-homohexamer for ammonium, it had been predicted earlier that the α-homohexamer would have a higher aminating activity than any heterohexamer composed of the two subunits (Bascomb and Schmidt, 1987).

Example 2 – Sequencing of Polypeptides and Polynucleotides

Amino-terminal sequencing of the mature subunits. An aliquot of a preparation of purified NADP-GDH α-subunit (120 pmol) and a partially purified preparation of NADP-GDH α-subunit (80 pmol) and β-subunit (50 pmol) were resolved by 8% (w/v) Tris-Tricine SDS-PAGE and electroblotted to a PVDF membrane (Immobilon-P^{SQ}, Millipore) as described by Plough *et al.* (Plough, M., A.L. Jensen, V. Barkholt [1989] *Anal. Biochem.* 181:33-39). To prevent *in vitro* acylation of the protein amino-terminal residues, all polyacrylamide solutions used in PAGE were treated with AG501-X8 mixed bed resin to

remove contaminating acrylic acid. An Applied Biosystems, Inc. model 470A gas phase sequencer was utilized for automated Edman degradation amino sequence analysis. The PTH-aa derivatives were identified by RP-HPLC. Protein sequence analysis of the electroblotted proteins was provided by the Interdisciplinary Center for Biotechnology

5   Research Protein Chemistry Core facility at the University of Florida.

The following N-terminal sequence was determined for the α-subunit: AVSLEEQISAMDATTGDFTA (SEQ ID NO. 5). The following N-terminal sequence was determined for the β-subunit: DATTGDFTAL (SEQ ID NO. 6). These sequences are identical to the ORF identified in the two NADP-GDH cDNAs and indicate the positions of

10   the internal cleavage sites utilized to remove the chloroplast targeting peptide sequences. The chloroplast targeting peptide sequences (or chloroplast-transit peptides) can be useful for cell compartment localization with these and other amino acid sequences. The polynucleotides encoding the chloroplast-transit peptides can be used with other polynucleotide sequences to encode chloroplast-transit peptides.

15   cDNA isolation and sequencing.   A pellet of *C. sorokiniana* cells stored at −70°C was resuspended 1 to 10 (w/v) in RNA breakage buffer: 0.1M Tris (pH8.5), 0.4M LiCl, 10 mM EGTA, 5 mM EDTA, 100 units/mL sodium heparin (Sigma, 100 units/mg), and 1 mM aurintricarboxylic acid (Sigma). The cell suspension was centrifuged at 7000g for 5 minutes at 4°C and the supernatant was discarded. The cell pellet was resuspended 1 to 10 (w/v) in

20   RNA breakage buffer and ruptured by passage through a French pressure cell at 20,000 p.s.i. The cell homogenate was collected in a disposable 50 mL conical tube containing 0.05 times volume 20% (w/v) SDS, 0.05 times volume 0.5 M EDTA (pH 8), 200 μg/mL proteinase K, and allowed to incubate at room temperature for 15 minutes. One-half volume of TE buffer (Tris 10mM:EDTA 1mM, pH 8.0) equilibrated phenol was added to the homogenate and

25   after a 3 minutes incubation a one-half volume of chloroform:isoamylalcohol (24:1,v/v) was added and mixed for 10 minutes on a wrist action shaker. The extracted homogenate was transferred to a 30 mL siliconized corex tube and centrifuged at 1000g for 10 minutes at 4°C. The upper aqueous phase was removed and repeatedly extracted with an equal volume of chloroform: isoamyl-alcohol (24:1, v/v), as described above, until the aqueous interface was

clear. After the final extraction, the aqueous phase was combined with an equal volume of 2X LiCl-Urea buffer (4 M LiCl, 4 M urea, 2 mM EDTA, 1 mM aurintricarboxylic acid; Sigma) and the RNA was precipitated on ice for 16 hours at 4°C. The RNA precipitate was centrifuged at 4000g for 20 minutes at 4°C and the resulting pellet was rinsed once with 1X

5   LiCl-Urea buffer and centrifuged again to pellet the RNA. The RNA pellet was solubilized in TE (pH 7.5) and an aliquot was quantified spectrophotometrically at 260 nm. After quantitation, the mRNA fraction was isolated from total cellular RNA using an oligo(dT) spin column kit. Poly(A)$^+$ RNA (50 μg) from each preparation was combined and utilized for the commercial production of a custom λUni-ZAP XR $C.$ $sorokiniana$ cDNA library

10   (Stratagene Cloning Systems, Palo Alto, CA).

The amplified λZAP library, containing 2 x 10^{10} pfu/mL, was plated on twenty 150 mm petri plates at 50,000 pfu per plate for a total of 1 x 10^6 pfu screened. The phage plaques were absorbed to duplicate Hybond-N 132 mm circular membranes and treated according to the plaque blotting protocol of Amersham (1985, Amersham International plc, Arlington

15   Heights, IL). Membranes were prehybridized in a common container in 200 mL of 2X PIPES (0.8 M NaCl, 20 mM PIPES, pH 6.5), 50% (w/v) formamide, 0.5% (w/v) SDS, 100 μg/mL denatured sheared salmon sperm DNA at 40°C. Blocked membranes were hybridized at 42°C in ten heat-sealable bags (four membranes/bag) in prehybridization buffer containing 1 x 10^6 cpm/membrane of a ^{32}P-labeled NADP-GDH 242 bp HCR cDNA probe

20   on a lab rocker. The membranes were washed three times in 200 mL of 0.1X SSC, 0.1% (w/v) SDS for 20 minutes per wash at 50°C. Duplicate membranes were wrapped in plastic wrap and exposed to Kodak X-Omat AR film at −70°C for 28 hours. Putative NADP-GDH cDNA plaques, detected on duplicate membranes, were cored from the plate and plaque purified by secondary and tertiary screenings with the 242 bp conserved region probe.

25   Putative NADP-GDH cDNA phage clones, selected in the primary screening, were combined and screened a second time with a ^{32}P-labeled 130 bp Eco RI/Bgl II cDNA fragment isolated from the 5′ terminus of the most complete 5′ end NADP-GDH cDNA clone. Ten plaque pure NADP-GDH clones were subcloned in pBluescript KS$^+$ (Stratagene) and transformed into $E.$ $coli$ DH5α F′ (Bethesda Research Laboratories, BRL) via an in $vivo$ excision protocol

provided by Stratagene. All plasmid isolations were performed as described by Kraft *et al.*
(Kraft, R., J. Tardiff, K.S. Krauter, L.A. Leinwand [1988] *Biotechniques* 6:544-547).
Sequence analysis revealed all ten clones were identical at their 3'-termini and differed by
varying degrees of truncation at their 5'-termini. The longest cDNA clone with a complete

5   3'-terminus designated pBGDc53 (SEQ ID NO. 7) was not long enough to encode either
subunit; therefore, the 5'-terminal sequences were determined by RACE PCR.

The 5'-terminal NADP-GDH cDNA sequences were cloned using a modified
anchored PCR procedure for the rapid amplification of cDNA ends (Frohman, M.A. [1990]
In D.H. Gelford, J.J. Snincky, T.J. White, eds, *PCR Protocols*, Academic Press, San Diego,

10   CA, pp 28-38; Jain, R., R.H. Gomer, J.J. Murtagh [1992] *Biotechniques* 12:58-59). A
mixture of poly(A)$^+$ RNA, used in the synthesis of the λZAP library, was utilized to clone
the 5' end of the NADP-GDH mRNA. One hundred nanograms of the mRNA mixture were
combined with 10 ng of a gene-specific primer (5'-CTCAAAGGCAAGGAACTTCATG-3',
SEQ ID NO. 8), designed to hybridize to the conserved region of NADP-GDH mRNAs,

15   heated for 5 minutes, and chilled on ice. First strand DNA synthesis was performed using
Superscript™ reverse transcriptase (BRL) according to the supplier's protocol. The
terminated reverse transcription reaction was treated with one unit of ribonuclease H for 20
minutes at 37°C, 5 minutes at 95°C, and extracted once with chloroform:isoamyl alcohol
(24:1, v/v). Excess primers and dNTPs were removed by centrifugation at 2000 rpm through

20   an Ultrafree-MC filterfuge tube (30,000 MW cutoff, Millipore) and the retentate was
concentrated to 10 µl on a Savant Speedvac. The first-strand synthesis products were
combined with 10 µL of tailing mix (1X tailing buffer [Promega Corp.], 0.4 mM dATP, 10
units terminal deoxytransferase) and incubated at 37°C for 10 minutes. The reaction mixture
was heated to 95°C for 5 minutes, diluted to 0.5 mL with TE (pH 8), and utilized as a cDNA

25   pool. A mixture of 5µL of the cDNA pool, 5 µL of Vent™ polymerase 10X buffer (New
England Biolabs), 200 µM of each dNTP, 25 pmol of a gene specific primer (SEQ ID NO.
8), 5 pmol of the poly(dT) adaptor primer (5'-
GGGTCGACATTCTAGACAGAATTCGTGGATCC(T)$_{18}$-3'; SEQ ID NO. 9), 0.2 units
Perfectmatch™ DNA polymerase enhancer (Stratagene), and 1 unit of Vent™ polymerase

(NEB) in 50 µL was amplified according to Jain *et al., supra*. The PCR products were purified away from the excess primers by centrifugation at 2,000 rpm through an Ultrafree-MC unit. The retentate was collected and subjected to two more rounds of amplification using a new nested gene specific primer at each step (5'-GGACGAGTACTGCACGC-3', SEQ ID NO. 10; 5'-GATCTCGGTCAGCAGCTG-3', SEQ ID NO. 11, respectively) and an adaptor primer (5'-GGGTCGACATTCTAGACAGAA-3'; SEQ ID NO. 12). PCR amplifications were performed in a Model 480 thermocycler (Perkin-Elmer Cetus), and all custom oligonucleotides were synthesized by the ICBR DNA synthesis facility, University of Florida. The standard PCR reaction mixture consisted of 10 µL of 10X Vent™ polymerase buffer, 100 µM of each dNTP, 0.4 units of Perfectmatch™, 50 pmol of each primer, 1 unit Vent™ DNA polymerase in a 100 µl reaction volume. The 5' RACE-PCR products were gel purified, subcloned into the *Sma*I site of pUC 18, and transformed into *E. coli* DH5α for further characterization. RACE PCR identified two 5' cDNA clones, which overlapped with the previously identified pBGDc 53 clone, that differed by a 42 nt insert identified in one clone designated pRGDc 60 (SEQ ID NO. 13) and lacking in the second cDNA designated pRGDc 61 (SEQ ID NO. 14).

Two additional cDNA clones lacking the RACE PCR polylinker, but possessing the complete 5'-termini corresponding to pRGDc 60 and 61 were constructed by RT-PCR amplification from mRNA using reaction conditions as described above and the gene specific primer pair (5'-CTTTCTGCTCGCCCTCTC-3', SEQ ID NO. 15, and SEQ ID NO. 11, above). The two PCR products were cloned into the *Sma*I site of pBluescript SK+ (Stratagene) and transformed into *E. coli* DH5α for further characterization. The cDNA clone that possessed the 42 nt insert was designated pGDc 63 (SEQ ID NO. 16) whereas the cDNA lacking the insert was designated pGDc 64 (SEQ ID NO. 17).

Full-length NADP-GDH cDNAs were constructed by restriction endonuclease treating pGDc 63 and 64 with *Eco*RI/*Apa*LI and gel purifying the resultant (264 bp; 222 bp, respectively) fragments. The gel purified fragments were ligated to a purified *Apa*LI/*Xho*I restriction fragment of pBGDc 53 and the full length ligation products (SEQ ID NO. 18; SEQ ID NO. 19) were gel agarose gel purified and utilized in subsequent PCR reactions.

Expression of α- and β-homohexamers in *E. coli*. Using the gel purified product (SEQ ID NO. 18), PCR mutagenesis was performed to remove the chloroplast targeting signal from the full-length cDNA and yield cDNAs encoding specifically the mature α- and β-subunits. Two sets of primer pairs were designed to synthesize α- and β-GDH subunit genes.

The following primer was designed to add a methionine to the amino terminus of the processed mature α-NADP-GDH subunit (alanine-41) to allow translation initiation and to generate a 5′ *Nde*I site for subcloning purposes: 5′-CATATGGCCGTCTCGCTGGAGGAG-3′ (SEQ ID NO. 20). The following second primer was designed to hybridize to the 3′ terminus of the template DNA at a position 20 nt 3′ of the endogenous TAA termination codon: 5′-GTTGGATTGCCGGTGAGCC-3′ (SEQ ID NO. 21).

The following primer was designed to add a methionine to the amino terminus of the processed mature β-subunit (aspartate-38) to allow translation initiation and to generate a 5′ *Nde*I site for subcloning purposes: 5′-CATATGGACGCCACCACCGGC-3′ (SEQ ID NO. 22). The second 3′ primer used in the PCR amplification was the 3′-terminus primer (SEQ ID NO. 21) described for the α-subunit amplification.

PCR cycling conditions were as follows: 95°C, 50 seconds; 64°C, 1 minute; 72°C, 1 minute 35 seconds (30 cycles). Primer, dNTP, Vent polymerase, and other reaction component concentrations were as previously described. The 1506 bp α-NADP-GDH subunit gene (SEQ ID NO. 23) and 1473 bp β-GDH subunit gene (SEQ ID NO. 25) PCR products were gel purified and given a 3′ adenine nucleotide overhang by incubating the purified fragment with 100 μM dATP and *Taq* polymerase for 15 minutes at 72°C. The modified PCR products were cloned into the PCRII T/A cloning vector (Invitrogen) and transformed into competent *E. coli* cells. Clones bearing the inserts were selected by blue-white screening, plasmid purified, and digested with *Nde*I/*Bam*HI to select for the proper orientation in the cloning vector. The selected plasmids were restricted with *Nde*I and *Bam*HI (*Bam*HI site provided by vector) and directionally cloned under the control of the IPTG inducible T7 polymerase promoter of pET 11a and pET 15b bacterial expression vectors (Novagen) linearized with *Nde*I/*Bam*HI, and transformed into DH5α. Transformants were

screened by *NdeI/Bam*HI restriction analysis and clones possessing the properly oriented α-
and β-subunit cDNAs (SEQ ID NO. 23; SEQ ID NO. 25) were selected, plasmid purified,
and transformed into *E. coli* BL21(DE3) for protein expression purposes.

        E. coli BL21(DE3) cells transformed with pET 11a-α-cDNA and pET 11a-β-cDNA

5   constructs were induced with 100 mM IPTG for 1 hour. Protein extracts from the induced
cells were tested by enzyme analysis for NADP-GDH activity, and the denatured proteins
were resolved by SDS gel electrophoresis, and visualized by coomassie staining. The
proteins expressed by the mature α-subunit cDNA (SEQ ID NO. 23) and the β-subunit cDNA
(SEQ ID NO. 25) have the amino acid sequences shown in SEQ ID NO. 24 (α-subunit) and

10   SEQ ID NO. 26 (β-subunit). The recombinant GDH subunits were verified by crossreactivity
with rabbit anti-*Chlorella* NADP-GDH antibodies.

        Under conditions not optimized for maximal induction, the *E. coli* cells, possessing
the α- and β-GDH cDNAs and induced with IPTG, showed 60- and 7,000-fold increases in
NADP-GDH activity relative to uninduced controls, respectively. The recombinant α- and

15   β-NADP-GDHs are currently being analyzed to verify kinetic and biochemical properties.

        The over-expression and assembly of the *C. sorokiniana* chloroplastic GDHs into
active enzymes provides proof that the DNA constructs engineered via PCR are transcribed
and translated into authentic proteins. The aforementioned constructs were then utilized for
cytosolic expression of the algal GDHs in transgenic plants.

20   Transformation of plants. A method for producing genetically transformed plants that
express increased levels of a specific GDH requires the introduction of a double-stranded
recombinant DNA molecule into the nuclear genome of a plant cell. The DNA molecule
must (1) contain a structural DNA for the GDH enzyme being introduced into the plant cell;
(2) possess a promoter which functions in plants to regulate the production of an RNA

25   sequence in a constitutive or tissue-specific manner by RNA polymerase enzyme; and (3)
have a 3'-untranslated region which functions to cause transcriptional termination and the
addition of polyadenylated nucleotides to the 3' end of the RNA. The resulting primary
RNA molecule is subsequently processed in the nucleus, a process which involves the

removal of intronic sequences and the addition of polyadenylate nucleotides to the 3' end of the mRNA.

Promoters which are useful in the present invention are those that can initiate transcription in a constitutive manner or in a tissue-specific manner where glutamate production or catabolism is desired. An example of a useful constitutive promoter is the CaMV enhanced 35S promoter that directs the synthesis of RNA in a tissue independent manner. Promoters which cause production of GDH specifically in seeds, stems, roots, leaves, or specific cell types in these tissues are useful in the present invention. For example, the seed-specific Phaseolin promoter is one such tissue-specific promoter. Thus native promoters for maize, wheat, barley, and rice may be obtained and used in the present invention as well as heterologous promoters from other organisms shown to function in a constitutive/tissue-specific manner.

Introns. Generally, optimal expression in monocotyledonous plants is obtained when an intron sequence is inserted between the promoter sequence and the structural gene sequence. An example of such an intron sequence is the HSP 70 intron described in WO 93/19189.

Polyadenylation signal. The DNA constructs of the present invention can possess a 3' untranslated region which functions in plants to direct the addition of polyadenylate nucleotides to the 3' end of the RNA. An example of a suitable 3' untranslated region is the polyadenylation signal of the *Agrobacterium* tumor inducing plasmid, i.e., nopaline synthatase (NOS) gene.

Plastid targeting sequence. The DNA constructs of the present invention can optionally contain a plastid targeting sequence. The plastid targeting sequence directs the import of the protein into the plastid, and is removed during importation. The plastid targeting sequence can be, but is not limited to, the native chloroplast targeting peptide (CTP) identified in the *C. sorokiniana* NADP-GDH full-length cDNAs which encode the precursor proteins. A fusion of a selected plastid targeting sequence and the mature α- and β-NADP-GDH subunit sequences can be made by standard procedures and used in the present invention. GDH subunits lacking these targeting sequences are typically found in the

cytoplasm of the cell. Such a cytosolic localized enzyme can be useful in capturing ammonium or glutamate compartmentalized in the cytosol of the cell.

GDH gene sources. The GDH gene used in the DNA constructs of the present invention can be any GDH gene. It is not limited to the *C. sorokiniana* GDH genes described above, although they are preferred. For example, a GDH gene from bacteria or fungi can be used. The examples provided use the α- and β-GDH genes of *C. sorokiniana*, but should not be interpreted in any way to limit the scope of the present invention. Individuals skilled in the art will recognize that various other genes as well as alterations can be made to genes and methods described herein while not departing from the spirit and scope of the present invention. For example, mutagenesis and routine screening can be implemented by techniques well known in the art to produce mutant variants that lack regulation by the cofactor NADPH.

Transient expression in maize protoplasts. In order to test the expression of the *C. sorokiniana* GDH subunits and their assembly into active enzymes in *Zea mays* cells, vectors were constructed to contain the CaMV E35S promoter, the coding sequence for the mature α-subunit (pMON21904) or β- subunit (pMON21905), the NOS 3'-untranslated polyadenylation region, and kanamycin resistance for selection in *E. coli*. The α- and β-subunit genes were isolated as a *Xba*I-*Eco*RI fragment from pET 11a-α-cDNA and pET 11a-β-cDNA, respectively. The GDH genes were ligated into the *Xba*I-*Eco*RI E35S promoter, NOS 3', kanamycin resistance bearing region of pMON22072 to give pMON21904, and pMON21905. The DNA constructs were electroporated into maize and wheat protoplast according to the method of Sheen *et al.* (*The Plant Cell* Vol. 3, 225-245).

Analysis of transformed maize protoplasts. Pelleted protoplast samples transformed with pMON21904 (α-subunit), pMON21905 (β-subunit), pMON21709 (kanamycin negative control DNA), and no DNA were thawed in 0.2 mL of GDH cell breakage buffer (Yeung *et al., supra*) on ice. The cells in each suspension were homogenized twice for 30 seconds, chilled on ice, and clarified at 14,000 rpm for 10 minutes. Cell extracts were assayed in the deaminating direction at 38.5°C according to Yeung *et al., supra*. Total protein content of the cell extracts was determined using the BioRad microprotein assay according to the

manufacturer's protocol. Activities were normalized against total protein content for comparisons among different preparations. One unit of GDH activity is defined as the amount of enzyme necessary to reduce 1 μmol of NADP per minute at 38.5°C.

Protoplasts transformed with the control vector pMON21709 (n=3) or protoplasts not transformed (n=3) had no detectable NADP-GDH activity. Protoplasts transformed with pMON21904 (n=3) expressed 3.31 Units mg^{-1} protein of GDH activity, whereas pMON21905 transformed protoplasts (n=3) 1.96 Units mg^{-1} protein.

The high level of activity observed for the protoplasts transformed with the cytoplasmic expressed *C. sorokiniana* α- and β-NADP-GDH genes provides evidence that the GDH subunits are expressed in heterologous plant systems. Additionally, expression levels demonstrate that the subunits are assembled into active enzymes. Generally, it would be readily apparent to persons of ordinary skill in the art that superfluous sequences added to the described sequences, or fragments of the nucleotide or amino acid sequences described herein, which result in polynucleotides or amino acid sequences that function similarly or equivalently to the sequences expressly described herein, should also be considered part of this invention. They can easily and routinely be produced by techniques well known in the art, for example, by time-controlled *Bal*31 exonuclease digestion of the full-length DNA, followed by expression of the resulting fragments and routine screening of the expression products as described in the foregoing example. In addition, it would be readily accepted by ordinarily skilled artisans that the function, property, or utility of the described sequences can be negatived by inserting mutations into the sequences by standard techniques and procedures. These mutations which, by implication, effectively serve to remove the property or function inherent in the sequences as described are hereby expressly included as part of the invention. For example, a clear distinction between the α- and β-subunits of the *C. sorokiniana* is the 11-amino acid polypeptide sequence at the N-terminus of the α-subunit, but absent in the β-subunit. This sequence can affect the affinity, specificity, and modulation of ammonium compounds by the enzyme. Therefore, it would be apparent that inserting (if absent) or removing (if present) the appropriate sequence, or its functional equivalent, to

effect a difference in certain characteristics of other GDH genes, or their products, would be easily carried out by those persons.

5   It should also be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.